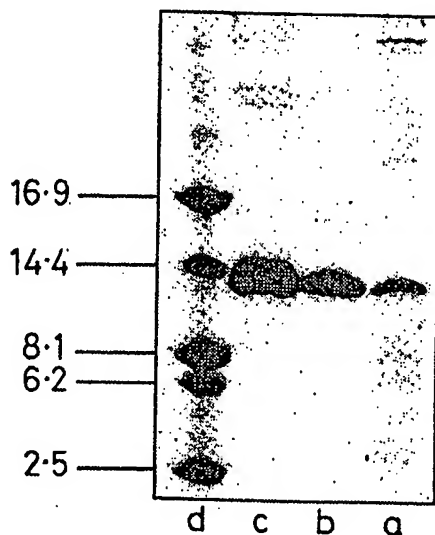




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(54) Title: PROTEIN BASED DELIVERY SYSTEM**(57) Abstract**

A delivery system, especially for delivery to targeted sites in the human or animal body, comprises capsids of the coat protein amino acid sequence of phage MS-2 or related phage, or a modification thereof which retains capsid-forming capability, or sufficient of said sequence or modification to retain capsid-forming capability, at least some of said capsids enclosing a moiety foreign to the genome of MS-2 or related phage.

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PROTEIN BASED DELIVERY SYSTEM

This invention relates to a protein-based delivery system and is particularly directed to the delivery of encapsidated foreign moieties, especially to targeted sites in the human or animal body.

There is increasing interest in the targeting of foreign moieties to the sites in the body where their activity is required. Thus it is important that drugs, particularly those having undesirable side effects, are delivered to the site where they are to act. Many other molecular species require to be delivered in a site specific manner, often to particular cells, for example polynucleotides (anti-sense or ribozymes), metabolic co-factors or imaging agents. One such system has been described by Wu *et al.*, J. Biol. Chem., 263, 14621-14624 and WO-A-9206180, in which a nucleic acid useful for gene therapy is complexed with polylysine linked to galactose which is recognised by the asialoglycoprotein receptors on the surface of cells to be targeted. However, there are many occasions, such as in the delivery of a cytotoxic drug, when it would not be satisfactory to use a delivery system in which the moiety to be delivered is so exposed. There is therefore a need to develop alternative delivery systems which have the flexibility to target a wide range of biologically active foreign moieties.

Co-pending UK patent applications no. 9114003.8 and 9201372.1 describe the modification of the coat protein of phage MS-2 as a presentation system for epitopic species, which may be included in a modified coat protein sequence or attached to the coat protein via a cysteine residue and optional further spacer. These applications relied on the ability of the coat protein of MS-2 and similar phages to be cloned and expressed in a bacterial host such as *E. coli* as largely RNA-free empty phage particles. Romaniuk *et al.*, (1987), Biochemistry 26, 1563-1568 have studied the relationship between the MS-2 coat protein and the RNA genome. It is apparent that, although RNA-free coat

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protein assemblies can be produced in E. coli, capsid formation in natural infections is triggered by coat protein interaction with a 19 base stem-loop (translational operator) in the RNA genome sequence. Talbot et al., 1990, Nucleic Acids Research 5 18, No. 12, 3521-3528 have synthesised the 19 base sequence and variations of this sequence and investigated the recognition and binding by the coat protein. It has been found that not only does the translational operator RNA signal exist as the stem-loop structure within the larger genomic RNA but that it is 10 also recognised as the short fragment of just 19 bases. This fragment has the ability to cause recombinant coat protein to bind specifically and self-assemble around it, resulting in recombinant capsids containing multiple copies of the RNA fragment.

15 According to the present invention there is provided a delivery system comprising capsids of the coat protein amino acid sequence of phage MS-2 or related phage, or a modification thereof which retains capsid-forming capability, or sufficient of said sequence or modification to retain capsid-forming 20 capability, at least some of said capsids enclosing a moiety foreign to the genome of MS-2 or related phage.

The foreign moiety is suitably attached to a portion of the RNA genome sequence of MS-2 or related phage capable of functioning as a translational operator for capsid formation, or 25 a variant thereof retaining the translational operator function. The RNA genome sequence was first defined by Fiers, Nature, 1976, 260, 500-517, and we have found that the 19-base stem loop (bases -15 to +4 relative to the start of the replicase gene) or a variant thereof, especially the variant 30 where cytidine is substituted at the -5 position, is the minimum requirement for function as the translational operator (see Talbot et al., 1990, Nucleic Acids Research 18, No. 12, 3521-3528). The foreign moiety may be attached directly to the operator sequence or via a spacer moiety, for example a series

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of uridine residues (suitably 6) to ensure that the foreign moiety does not interfere with the operator function.

According to a preferred form of the invention the coat protein amino acid sequence has been modified to provide a site
5 suitable for attachment thereto of a targeting moiety. The invention includes capsids having such a site for subsequent attachment of a targeting moiety and capsids to which the targeting moiety has already been attached.

The coat protein amino acid sequence is preferably that
10 derived from phage MS-2, but it may also be derived from related RNA-phages capable of replication in *E. coli*, such as phages R17, fr, GA, QB and SP. Such RNA-phages of physical structure similar to that of MS-2 will contain some chemical variation in the amino acid residues of the coat protein and are thus
15 conservatively modified variants of MS-2 coat protein. While it is believed at present that substantially the entire coat protein may be required for capsid assembly, deletions and/or insertions are also possible whilst still retaining capsid-forming capability. Proteins having such modified
20 sequences are included within the scope of the invention.

The three-dimensional structure of the MS-2 phage particle has been published by Valegard *et al.*, (Nature, 1990, 345, 36-41). The published data show that, firstly, the structure of the coat protein is not related to the eight-stranded β -barrel
25 motif found in all other spherical RNA virus subunits whose structures are known at the present time. Secondly, although the coat protein exhibits quasi-equivalent inter-subunit contacts, there are no other devices, such as extended arms of polypeptide, helping to secure each protein conformer. The coat
30 protein structure can be viewed in terms of three separate regions. These are not domains in the usual sense but could represent independent folding units. These regions are residues 1-20, which form the β -hairpin structure which protrudes from the surface of the phage forming the most distal radial
35 feature. This region is followed by residues 21-94 which form

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five β -strands including the "FG-loop" which is the site of the only major conformational change between quasi-equivalent conformers. These β -strands are then followed by two α -helices, residues 95-125, which interdigitate to secure dimers of the coat protein sub-units. Valegard *et al.* are concerned solely with the physical structure of the MS-2 virus and do not attempt to elucidate the mode of action of the virus.

Co-pending UK patent application No. 9114003.8 describes the introduction of a cysteine residue into the N-terminal protruberant β -hairpin of the coat protein (with removal of the cysteine residues present externally of the N-terminal protruberant β -hairpin). Such a cysteine residue provides a preferred site for attachment thereto of a targeting moiety. The resultant coat protein has therefore been so modified in the region of amino acid residues 1 to 20, such numbering being with reference to the entire coat protein sequence of MS-2 as published by Fiers, Nature, 1976, 260, 500-507. Preferably the modification to introduce the cysteine residue is towards or at the middle of the hairpin region. It is preferred to introduce the cysteine in the region of the glycine 13 and 14 residues of the coat protein. The cysteine residues to be removed which are external of the β -hairpin are found at positions 46 and 101. They may be removed by any convenient conventional genetic engineering technique, suitably by site-specific mutagenesis.

In a preferred method of removing the unwanted cysteine residues, two mutants of the MS-2 coat protein, one singly mutated at cys 46 and one singly mutated at cys 101 may be obtained by standard commercially available techniques for site specific mutagenesis and the corresponding cDNA sequences introduced into standard expression vectors, which vectors are subjected to digestion with restriction enzymes to obtain separately the DNA fragment containing the mutated cys 46 site and the corresponding fragment containing the mutated cys 101 site, the fragments being subsequently ligated to give a doubly-mutated coat protein cDNA. The doubly-mutated cDNA may

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then be subjected to site-directed mutagenesis using standard methods to introduce a cysteine residue in the β -hairpin region.

Alternative modification of the coat protein which enables targeting of the encapsidated moiety may include insertion of peptide sequences in the protruberant β -hairpin of the MS-2 coat protein as described in co-pending UK patent application no. 9201372.1.

The cysteine residue, or alternative modification site, can be further linked to a targeting moiety with or without interposition of a further spacer moiety. An example of such a targeting moiety is a galactose residue which can be used to direct the capsids to interact with specific cell surface receptors and thus carry the foreign moiety within the capsids to and/or into specific cells. Other possible targeting moieties are other cell surface receptor ligands or monoclonal antibodies. Suitable receptors for the targeting moieties are the asialoglycoprotein receptor and the receptor for melanocyte stimulating hormone.

Suitable spacer moieties, if employed, are selected from known commercially available heterobifunctional crosslinking reagents which couple with the exposed cysteine thiol group. Examples of such cross-linkers are m-maleimidobenzoyl-N-hydroxy-sulfosuccinimide ester, N-succinimidyl-(4-iodoacetyl)amino-benzoate and N-succinimidyl-3-(2-pyridyldithio)propionate. The choice of crosslinker will depend on the targeting moiety and its size. Thus larger molecular species may require longer crosslinking moieties to minimise steric hindrance. The crosslinker may be linked first to the cysteine residue or first to the targeting moiety.

Alternatively the thiol function (or other derivatisable group) can be introduced into wild type, empty capsids of MS2 coat protein using suitable heterobifunctional chemical reagents such as N-succinimidyl S-acetylthioacetate (SATA).

The foreign moiety held within the capsids can vary widely and include genes and gene fragments, ribozymes, anti-sense

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messages or cytotoxic and chemotherapeutic agents intended for such purposes as anti-sense gene therapy or selective killing of target cells.

5 The form in which the foreign moiety is held within the capsids will depend on the release properties required. For release at the targeted site it will be important to ensure that the right conditions prevail, for example to permit cell localisation and internalisation via receptor mediated endocytosis.

10 The capsids may suitably be obtained by first obtaining empty MS-2 capsids, for example by expression of vectors containing coat protein cDNA in E. coli as described in co-pending UK application No. 9201372.1. The MS-2 capsids may be of wild type MS-2 coat protein or have been modified, for
15 example to introduce a cysteine site as described in co-pending UK application No. 9114003.8. The capsids are then disassembled, for example, at acid pH (e.g. using acetic acid), before reassembly suitably at raised pH, e.g. pH 7. in the presence of the desired foreign moiety linked to an RNA sequence
20 capable of functioning as the translational operator in the reassembly of the coat protein around the RNA sequence and foreign moiety. Other methods of disassembly may be used, for example in the presence of urea. It is also contemplated that the capsids enclosing the foreign moiety may be obtained by
25 random incorporation of the moiety in the capsids.

The RNA sequence may be obtained by biochemical methods from the complete MS-2 RNA genome. Alternatively, the RNA sequence is obtained by chemical synthesis, for example as described by Usman et al., (1987), J. Am. Chem. Soc., 109, 7845-7854.
30 Chemical synthesis is preferred as it enables ready addition of any spacer moiety and linking to the foreign moiety to be delivered.

It will be apparent that there are several advantages in using MS-2 and related phages as a presentation system. Thus
35 the empty coat protein capsids can be readily expressed in

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comparatively high yield in *E. coli* and the product is easily purified (see R. A. Mastico et al. J. Gen. Virol. (1993) 74, 541-548 the contents of which are herein incorporated by reference). It has been found that the assembled capsids show
5 considerable stability with respect to a range of temperatures, pH and ionic strength.

The invention will now be described by way of example.

A) Preparation of MS-2 coat protein capsids

The coat protein of MS-2 was obtained by growing phage MS-2,
10 purifying the RNA, followed by oligonucleotide primer directed reverse transcription to produce single-stranded cDNA which was converted to double stranded cDNA using oligo primers and Klenow polymerase. The cDNA was then subcloned into an expression vector pGLW11 (Smith, M.C.M., Czaplewski, L.G., North, A.K.,
15 Baumberg, S. and Stockley, P.G. (1989) Mol. Microbiol. 3, 23-28,) placing the coat protein under the control of the inducible *lac* promoter.

The pGLW11 expression vector was expressed in *E. coli* and the cellular proteins obtained, purified and characterised as
20 follows:

Standard laboratory strains of *E. coli* were transformed (to ampicillin resistance) with the expression plasmid carrying the recombinant MS-2 coat protein gene. Rapidly growing cultures of these transformants in rich media were induced by addition of
25 isopropyl- β -thiogalactoside (IPTG) to a final concentration of 1mM when the O.D.₆₀₀ of the culture was between 0.4-0.6. Cell growth was continued overnight before the cells were harvested by centrifugation, resuspended in neutral buffer, sonicated to lyse the cells, followed by centrifugation to separate the
30 supernatant (containing the expressed recombinant products) and cellular debris. The supernatant was fractionated by ammonium sulphate precipitation, the pellet of product being resuspended in buffer before being purified on the basis of size by either sucrose density gradients or gel filtration chromatography or by
35 immuno-affinity chromatography (Mastico et al. (1993)). The

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product was obtained in the form of capsids which were subsequently disassembled by the addition of 2 volumes of glacial acetic acid.

B) Preparation of RNA genome oligonucleotides

5 Four oligonucleotides were prepared by solid phase chemical synthesis using 2'-silyl-protected phosphoramidite starting materials as described by Usman *et al.*, (1987), J. Am. Chem. Soc., 109, 7845-7854 and Talbot *et al.*, (1990), Nucleic Acids Research, 18, No. 12, 3521-3528.

10 a) Containing nucleotides -15 to +4 of the RNA genome of MS-2 with a cytidine introduced at position -5, i.e. a sequence encompassing the translational operator (described hereinafter as "MS-2C").

15 b) MS-2C carrying a 5' biotin residue as a model foreign moiety;

c) As for a), but with 6 uridine residues linked at the 5' end of the oligonucleotide (described hereinafter as "MS-2C+6U") also carrying as a model foreign moiety a 5' biotin residue.

20 d) As for a) but with a 3' extension of 22 deoxynucleotides which are complementary, i.e. anti-sense, to the first 22 nucleotides of the mRNA for the HIV-1 Tat protein.

The biotin group in b) and c) was introduced as follows:

25 500mg of "DMT biotin-C6-PA" (a dimethoxytrityl-protected biotin-C6-spacer-phosphoramidate reagent available from Cambridge Research Biochemicals Ltd., Cheshire, UK) was dissolved in 0.6ml of anhydrous acetonitrile. The synthesis of both MS-2C + biotin and MS-2C+6U+biotin was carried out on an Applied Biosystems Model 391 DNA synthesiser on a 1µMol scale.

30 Results of the syntheses with biotin:

	Overall Yield	Average Stepwise Yield
MS-2C+biotin	62.4%	97.5%
MS-2C+6U+biotin	38.9%	96.2%

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The solid supports from all four syntheses were transferred into clean vials and treated with HPLC grade methanol saturated with ammonia for 24 hours at room temperature. The supernatants were transferred to fresh vials and dried down using a stream of nitrogen gas.

The resulting pellets were then resuspended in t-butylammonium fluoride in THF, and incubated at room temperature for 24 hours, and then quenched using an equal volume of 1M ammonium acetate.

The quenched deprotection reactions were then desalted using Pharmacia NAP 25 columns. The columns were equilibrated with 25mls 0.2X TBE and 2.0mls of sample was added to the columns, with 0.5ml fractions being collected. Once all the material had been eluted, the columns were re-equilibrated as before and the second half of the sample was desalted likewise. A fresh column was used for each RNA oligonucleotide.

The size of each product was confirmed by 3' radiolabelling and chromatography over polyacrylamide sequencing gels. Autoradiography recorded single dominant radioactive species with expected mobilities.

C) Reassembly of capsids in the presence of
oligonucleotide-biotin and MS-2C-anti-Tat

Reassembly of the disassembled MS-2 capsids described in A) above was carried out by raising the pH from 2.4 to 7.0 in the following circumstances and in each case in the presence of an avidin-fluorescein complex (Pierce Europe BV, Holland).

- i) MS-2 coat protein only (no oligonucleotide present)
- ii) MS-2 coat protein plus MS-2C
- iii) MS-2 coat protein plus MS-2C+6U-biotin
- iv) MS-2 coat protein plus MS-2C-biotin.

The resulting capsids were separated on an HPLC gel filtration column and the results are shown in Figs. 1 to 6 where Fig. 1 represents the trace obtained using wild type MS-2 capsids which had not been disassembled and reassembled and Fig. 2 represents molecular weight standards. The peak eluting

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after approximately 19 minutes corresponded to the assembled MS-2 capsids. Figs. 3 to 6 represent the traces obtained for the mixtures i), ii), iii) and iv) respectively. It will be seen that while i) shows only a small indication of capsid formation, ii) shows a greatly increased amount of capsid as expected while iii) and iv) show retained capsid formation in the presence of avidin-fluorescein which will complex with the biotinylated oligonucleotides.

The presence of the avidin-fluorescein-biotin complex within the capsids was demonstrated by measuring the fluorescence intensity (resulting from fluorescein) in the fractions corresponding to the capsid peaks in Figs. 3, 4 and 5 (i.e. for i), ii) and iii) above) plus additionally a mixture:

v) MS-2 coat protein plus 1:1 mixture of MS-2C+6U-biotin and MS-2C (no biotin) plus avidin-fluorescein complex.

The results are shown in Fig. 7 and it will be seen that iii) showed the presence of fluorescence in the fractions corresponding to the capsids while v) showed the presence of a reduced level of fluorescence when the biotinylated oligonucleotide was diluted 1:1 with non-biotinylated oligonucleotide. The avidin-biotin complex is thus shown as being within the capsids.

MST WT capsids are reassembled with the (MS-2C-anti-Tat) as follows:

25 a) Reassembly

MS2 WT CP was purified as usual, but concentrated to 10mg/ml by spinning down at 35k rpm for 6h at 4°C. 0.4ml of the MS2 (10mg/ml) was added to 0.8ml of glacial acetic acid and kept on ice for 30 min. The precipitates were removed by centrifugation at 6500rpm, 4°C for 20 min and the supernatant passed over a NAP-25 column equilibrated with 1mM acetic acid. Both the protein and the oligonucleotide were mixed with 10x TMK buffer (100mM Tris, 80mM KCl and 10mM MgCl₂) and kept on ice for 1h. The MS2 CP was added to the MS-2C-anti-TAT solution at molecular ratios from 180:1 to 2:1 and the mixture incubated at 37°C for

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1h and then RT for 6h. The reassembled capsids were stable at 4°C for 2 weeks.

b) Analysis of the reassembled capsids

In order to confirm that the reassembled particles had encapsidated the test oligonucleotide capsids were separated from other components by HPLC gel filtration chromatography as described above. The peak corresponding to capsids was then phenol extracted, the nucleic acids precipitated with ethanol, the precipitate radiolabelled with ³²P and electrophoresed over a denaturing polyacrylamide gel. The results showed that a nucleic acid fragment with identical mobility to the starting material had been recovered from the HPLC column confirming encapsidation. Finally, we used transmission electron microscopy (as described above) to investigate the reassembled particles. This showed that the bulk of the input coat protein had reassembled into capsids of similar size and symmetry to the wild type phage.

c) Eukaryotic cell transfection

Human HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Twenty four hours before transfection the cells were plated out at 1 x 10⁶ cells per 75cm² flask. The medium was changed half an hour before the transfection.

The cells were transfected with 5µg LTR Cat and 5µg pSVTat (1) in 0.5ml of calcium phosphate-DNA coprecipitate (2). After 16 hours the cells were washed with PBS and fresh medium was added. The cells were then incubated for 24 hours before harvesting. The transactivation of LTRCat by Tat was challenged with the antisense oligo directed against the first 22 bases of the Tat mRNA. This was attached to the 3' end of the MS2 RNA stem loop (-15 to +4) to direct the reassembly of the capsid around the oligo.

(5'-ACA-UGA-GGA-UUA-CCC-AUG-U--TAC-CTC-GGT-CAT-CTA-GGA-TTG-3')

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D) Preparation of cysteine or thiol-modified coat protein capsids

Cystine modified MS-2 coat protein was produced as follows:

Site directed mutagenesis and standard techniques were used
5 to produce amino acid mutants at coat protein positions 46 and 101.

Mutants were selected having either cysteine at position 46 substituted by serine or cysteine at position 101 substituted by serine.

10 Each single mutant DNA was expressed in E. coli to demonstrate the ability to self assemble.

The ser 46 single mutant from step A) was introduced into standard coat protein expression vector ptacACP and digested with SacI and XbaI restriction enzymes and the longer backbone
15 fragment so obtained treated with calf intestinal phosphatase and then purified on agarose or polyacrylamide gels before electroelution and precipitation.

The ser 101 mutant was treated likewise with omission of the phosphatase treatment. The smaller fragment containing the
20 C-terminal portion of the coat protein gene was purified by gel electrophoresis.

The large fragment containing the mutated cys 46 site and the small fragment containing a mutated 101 site were ligated by standard methods. The recombinant molecules thus obtained were
25 used to transform E. coli TGI to ampicillin-resistance and positive colonies checked for double mutation by DNA sequencing.

The doubly-mutant ser 46/101 coat protein cDNA from step B was introduced into an M 13 sequencing vector by standard subcloning methods, a single stranded template for site-directed
30 mutagenesis generated and a cysteine residue introduced at gly 14 using the commercially available site specific mutagenesis protocol based on nucleotide phosphothioates. There was thus obtained mutated cys 14 Ser 46/101 coat protein cDNA.

The isolated mutated cDNA was expressed in E. coli to
35 confirm the capsid-forming ability of the recombinant protein.

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The cys 14 ser 46/101 coat protein cDNA of C) above was introduced to expression vector pTAC-CP and the resultant plasmid used to transform *E. coli* strain TGI in accordance with standard procedure. The cys 14 ser 46/101 coat protein was then
5 produced according to the following protocol.

5 x 5ml (2TY media with 100µg/ml ampicillin) cultures of single colonies picked from transformation plates were grown for approx. 4hrs at 37°C and then used to inoculate 5 x 500ml flasks of 2TY plus ampicillin and the cultures were grown at 30°C.
10 When the cultures reached OD₆₀₀ approx. 0.45 protein production was induced by adding 1mM IPTG. Cells grown overnight were then centrifuged at 3k rpm, 30 mins, 4°C in a Beckman JA14 rotor.

The resulting pellets were resuspended in 50mM Hepes, 100mM NaCl, 10mM dithiothreitol (DTT), 5mM EDTA and 1mM phenylmethyl
15 sulphonyl fluoride (PMSF), and the cells lysed by sonication. The cell lysate was then centrifuged at 15k, 20 mins, 4°C in a Beckman JA20 rotor and the supernatant passed down a NAP-25 column (Pharmacia) to change buffers to 20mM NaPi (sodium phosphate-based buffer) pH 7.0. 1ml fractions were collected
20 from the NAP column, the MS-2 coat protein containing fractions (nos. 2 to 5 inclusive) added to an anti-MS-2 coat protein immunoaffinity column and the sample allowed to bind for 1 hour at room temperature with gentle agitation.

The column was washed with 20mM NaPi pH 7, then 10mM
25 NaPi/100mM NaCl pH 7. The sample was eluted with 20mls 20mM acetic acid/200mM NaCl approx. pH 2.7 and the first 4mls collected.

The pH was immediately adjusted by titration with 1M Tris.HCl pH 9 to pH 7-7.4 and the mixture centrifuged at 30k rpm,
30 4°C overnight (approx. 15 hrs) using a Beckman SW.55Ti rotor. The supernatant was decanted and the MS-2 protein pellet resuspended in a small volume of the required buffer.

Homogenous cys 14 modified capsids were obtained which were tested for their ability to react with an activated galactose
35 reagent as described in E) below.

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SDS-PAGE of the resultant immunoaffinity purified cys 14 modified capsids showed essentially a single component of the expected molecular weight. This result is shown in Fig. 8 where lane a) shows the cys 14 modified capsids, lanes b) and c) show wild type capsids respectively immunoaffinity purified and sucrose density purified and lane d) gives molecular weight standards.

Fig. 9 shows an electromicrograph of the immunoaffinity purified cys 14 modified capsids showing the presence of assembled particles similar to those produced by wild type coat proteins.

Thiol groups can also be introduced into wild type-assembled capsids using heterobifunctional reagents such as SATA, as follows:

2mg of N-succinimidyl S-acetylthioacetate (SATA, Pierce Immunotechnology) was completely dissolved in 0.5ml of dimethylformamide (DMF) by shaking, and 20 μ l aliquots stored at 4°C. The purified MS2 WT CP was passed over a NAP-25 column equilibrated with 0.05M phosphate buffer (PB, pH 7.5) immediately before conjugation. 1ml of MS2 CP (1mg/ml) was mixed with 20 μ l of SATA and kept at RT for 1h (SATA:MS2 = 50:1). The solution was then deacetylated with 0.1ml of freshly prepared hydroxylamine-HCl (25mg in 0.5ml H₂O) at RT for 2h. The MS2 derivative was separated from the reagent and by-products by desalting over a NAP-25 column equilibrated in 0.1M PB pH 7.5.

The stoichiometry of SATA groups introduced into wild type MS2 coat protein was determined either by modification with DTNB (as described below) or ³H-iodo-acetic acid according to standard methods. The results suggest that approximately two new thiols per CP monomer are introduced by the SATA modification. Both new thiols were completely modified by treatment with activated galactose, as described below.

E) Reaction of cysteine or SATA modified protein with activated galactose

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In order to test the reactivity of the cys 14 modified MS-2 capsids, a halogen-activated galactose was prepared as follows:

To a stirred solution of p-aminophenyl β -D-galactopyranoside (0.54g; 2 mmole) in water (4ml) and ethanol (6ml) was added
5 iodoacetic anhydride (0.9g; 2.5 mmole) at room temperature. After 2 hours, the reaction was concentrated to dryness and the residue washed with ether (2 x 10ml). Crystallisation from ethanol gave the product as needles (0.7g; 80%), mp 158-160°C.

The reaction of the cys 14 modified capsids with the
10 activated galactose was assayed using Ellman's reagent (dithionitrobenzoate, DTNB) which gives a characteristic absorption at OD₄₁₂ on reaction with free -SH groups. Figure 10 shows a control curve for reaction of free cysteine with DTNB. The control curve was obtained using:

15 100 μ l sample.
100 μ l DTNB 4mg/ml in 100mM Na₂HPO₄ pH8 ("buffer 1").
5ml "buffer 1".

The mixture was left at room temperature for 15 minutes after adding DTNB and then the OD₄₁₂ recorded.

20 Figure 11 shows the curve obtained when cysteine (4mM) was mixed with from 1 to 10mM activated galactose and left to stand at room temperature for 1 hour, followed by assay of these 100 μ l aliquots as described above using DTNB.

25 The cys 14 modified coat protein was reacted with DTNB as follows:

Purified cys 14 modified capsids were resuspended in buffer 1 containing 1mM EDTA to a final concentration of 400 μ g/ml. The following individual experiments were set up.

- 30 1) 100 μ l protein sample, 100 μ l 5mM activated galactose.
2) 100 μ l protein sample, 90 μ l buffer 1, 10 μ l 5mM activated galactose.
3) 100 μ l protein sample, 100 μ l buffer 1.

Each was left stirring at room temperature for 1 hour before addition of 200 μ l DTNB 4mg/ml in ethanol to the stirring
35 solution. OD₄₁₂ was recorded after 15 minutes and the results

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are shown in Table 1 below. The number of free thiols decreased with increasing exposure to the galactose reagent, confirming that the cys 14 capsids had been derivatised with galactose. Similar results were obtained with SATA - modified wild-type capsids.

Table 1

	Sample	O.D. 412nm
10	Buffer blank	0.0
	1) 100µl MS2-cys; 100µl gal	0.031
	2) 100µl MS2-cys; 90µl buffer; 10µl gal	0.080
	3) 100µl MS2-cys; 100µl buffer	0.118
15	F) Linking of cys 14 modified coat protein to immunogenic peptide.	

The purified cys 14 modified capsids were linked as described below to HA10, a 10-mer peptide sequence encompassing a nonapeptide epitope derived from the haemagglutinin of the human pathogen influenza virus and having an N-terminal cysteine residue extension, which 9-mer sequence YPYDVPDYA has been identified as containing one of the antigenic determinants by Wilson *et al.*, Molecular and Cell Biology, May 1988, 2159-2165 and Cell, 37, 1984, 767-778. The procedure involved an initial crosslinking step to form a disulphide linkage which was then oxidised.

The following reagents were employed to make up four test reaction mixtures:

- 2µg cys 14 modified capsids (about 3µl) ("cys bridge")
- 1µl 1M Tris.HCl pH8, 10mM EDTA ("buffer 2")
- 17µg HA9 peptide (about 2µl) ("peptide")
- 1µl 2-mercaptoethanol ("βME")

The following four test mixtures were prepared, in each case made up to 10µl with water:

- 1) cys bridge + buffer 2 + βME

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- 2) cys bridge + buffer 2
- 3) cys bridge + buffer 2 + β ME + peptide
- 4) cys bridge + buffer 2 + peptide

The mixtures were incubated for 1 hour at room temperature.

5 There was then added 1 μ l of a mixture of 0.37M sodium tetrathionate and 1.6M sodium sulphite (which had been freshly prepared in accordance with the method of Morehead *et al.*, Biochem., 23, 1984, 2500). The mixtures were left overnight at room temperature.

10 The mixtures were analysed using a PAGE Schagger System (Schagger *et al.*, 1987, Anal. Biochem., 166, 368-379), blotted onto nitrocellulose paper using a Bio-Rad Western blotting apparatus, with a transfer buffer of 39mM glycine, 48mM Tris, 0.1% (w/v) sodium dodecyl sulphate (SDS) and 20% methanol for a
15 transfer time of 1 hour at 450mA.

The blots were washed with phosphate buffered saline (PBS) pH 7.6 containing Tween 20 (polyoxyethylene sorbitan monolaurate - 3ml per litre PBS) to equilibrate. They were then incubated for 1 hour at 37°C with 35ml PBS-Tween plus 0.5% (w/v)
20 bovine serum albumin (BSA), washed 6 x 5 min. with 200ml PBS-Tween and subsequently incubated overnight at 4°C with 35ml PBS-Tween + 0.5% (w/v) BSA together with 100 μ l mouse anti-HA9 monoclonal antibody (obtained from Balcore Co., Berkley, USA). There then followed washing with PBS-Tween (6 x 5 min. - 200ml)
25 and incubation for half an hour with 35ml PBS-Tween + 0.5% (w/v) BSA together with 50 μ l goat anti-mouse IgG horseradish peroxidase (HRP) conjugate. After further washing (6 x 6 min. - 200ml PBS-Tween), the gel was excited by luminol Western blotting reagents (Amersham) and visualised.

30 The results showed that only a single band in the lane corresponding to sample number 4 cross-reacted with the anti-HA9 antibody. This is the expected result, samples 1-3 being negative controls. Thus it is possible to couple linear peptide fragments to cys 14 capsids using these methods.

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G) Covalent cross-linking of cys 14 modified coat protein to an enzyme or targeting ligand protein.

The purified cys 14 modified capsids described in E) above were covalently linked via a maleimide group to the enzyme
5 horseradish peroxidase (HRP) as follows:

HRP-maleimide conjugate (Pierce Europe BV, Holland), cys 14 modified capsids and β ME were used to make the following mixtures, each of which was made up to 100 μ l with 100mM NaPi, pH 7.2:

- 10 1) 20 μ g HRP-maleimide plus 1 μ l β ME
 2) 20 μ g cys 14 modified capsids plus 1 μ l β ME
 3) 20 μ g cys 14 modified capsids plus 20 μ g HRP-maleimide

Sample 3) was left for 1 hour at room temperature and then β ME added to quench any remaining thiols. Samples 1-3 were then
15 fractionated by HPLC gel filtration chromatography on PW 3000, 2 x 30cm columns, in 100mM NaPi, pH 7.2 at a flow rate of 0.5ml/min. Fractions (1 min. - 0.5ml) of the eluate were then assayed for HRP activity using the commercially available kit (ABTS reagent, Pierce), enzyme activity being estimated by
20 observing the increased absorbance of solutions at 410nm. The data showed a significant increase over background levels in fractions corresponding to the OD₂₈₀ peak of cys 14 assembled material of sample 3.

H: Coupling of SATA-modified MS2 WT CP to
25 maleimide-activated HRP.

Maleimide-activated HRP was purchased from Pierce at a concentration of 1mg/ml. 1ml of SATA-modified MS2 was reacted with 0.1mg HRP at RT for 6h and then transferred to 4°C for storage. Samples passed over HPLC gel filtration columns were
30 analysed to determine the efficiency of the conjugation by both enzyme assay for HRP and Western Blots with anti-CP antibodies, which were consistent with formation of a covalent cross-link between the capsid and HRP.

I: Coupling of SATA-modified MS2 WT CP to
35 maleimide-activated transferrin (TF).

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MS2 was modified with SATA using the same methods as described above. TF was then activated for reaction as follows: 1mg of transferrin (TF) was dissolved in 0.5ml of 0.05M PB, pH7.5, and 0.5 mg of Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) dissolved in 30 μ l of PB, and then the two solutions were mixed and kept at 37°C for 30 min with gentle shaking. The precipitates were removed by spinning at 3k rpm for 10 min at 4°C and the supernatant passed over a NAP-25 column equilibrated in 0.05M PB.

Conjugates were made as follows: 1 ml of MS2-SATA (0.75mg/ml) was added to 1ml of maleimide-activated TF (0.44mg/ml) in a solution flushed with nitrogen gas and kept at RT for 6h then stored at 4°C. Conjugates appeared stable over a period of at least one month at 4°C. The samples were analysed by HPLC chromatography and Western blotting with both anti-CP and anti-transferrin antibodies, as described above. The results confirmed formation of covalent cross-links between the capsids and transferrin.

20 H) Cell Entry of MS-2

Wild type recombinant MS-2 RNA-empty capsids prepared as described in A) above were allowed to react with rabbit polyclonal anti-MS-2 serum and then incubated for one hour with mouse macrophages. The macrophages normally function by binding to Fc portions of immuno-complexes via a cell surface receptor which is then endocytosed. The experiment thus sought to demonstrate cell entry of MS-2 particles via endocytosis. The results were analysed by embedding the macrophage cells in a plastic block followed by thin sectioning and various staining procedures. The results are shown in Figs. 12 to 15. Figs. 12 and 13 show macrophage sections negatively stained. 300Å particles (MS-2 capsids) can clearly be seen in essentially all the sections viewed. Figs. 14 and 15 show similar sections which have been treated with anti-rabbit peroxidase which then stains the anti-MS-2 antibodies (black dots in photograph).

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Both 300Å and larger aggregates are heavily stained and in the last panel the staining is clearly dispersing within the cell as would be predicted following dissociating of the complex in the lysosome.

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CLAIMS

1. A delivery system comprising capsids of the coat protein amino acid sequence of phage MS-2 or related phage, or a modification thereof which retains capsid-forming capability, or
5 sufficient of said sequence or modification to retain capsid-forming capability, at least some of said capsids enclosing a moiety foreign to the genome of MS-2 or related phage.
2. A delivery system according to claim 1 wherein the foreign
10 moiety is attached directly or indirectly to a portion of the RNA genome oligonucleotide sequence of MS-2, or related phage, capable of functioning as a translational operator for capsid formation, or a variant thereof retaining the translational operator function.
- 15 3. A delivery system according to claim 2 wherein the foreign moiety is attached directly or indirectly to bases -15 to +4 of the RNA genome oligonucleotide sequence of MS-2 or a variant thereof.
4. A delivery system according to claim 3 wherein the base at
20 position -5 has been substituted by cytidine.
5. A delivery system according to claim 2, 3 or 4 wherein a spacer moiety is included between the genome oligonucleotide sequence and the foreign moiety.
6. A delivery system according to any one of the preceding
25 claims wherein the coat protein amino acid sequence has been modified to provide a site suitable for attachment thereto of a targeting moiety.
7. A delivery system according to claim 6 wherein the site comprises a cysteine residue.
- 30 8. A delivery system according to claim 7 wherein the cysteine residue has been introduced to the N-terminal protruberant β -hairpin of the coat protein.

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9. A delivery system according to claim 6, 7 or 8 wherein a targeting moiety is directly or indirectly attached to the coat protein.
10. A delivery system according to claim 9 wherein the targeting moiety comprises galactose.
11. A delivery system according to any one of the preceding claims wherein the foreign moiety is selected from genes, gene fragments, ribozymes, anti-sense oligonucleotides, cytotoxic agents and chemotherapeutic agents.
12. A method of preparing a delivery system as claimed in any one of the preceding claims comprising disassembly of capsids of at least a capsid-forming sufficiency of the coat protein amino acid sequence of phage MS-2, or related phage, or a modification thereof and reassembly in the presence of the foreign moiety linked directly or indirectly to a portion of the RNA genome oligonucleotide sequence of MS-2, or related phage, capable of functioning as a translational operator for capsid formation, or a variant thereof retaining the translational operator function.
13. A method according to claim 12 wherein the disassembly is carried out at acid pH.
14. A method according to claim 12 or 13 wherein the reassembly is carried out at increased pH.

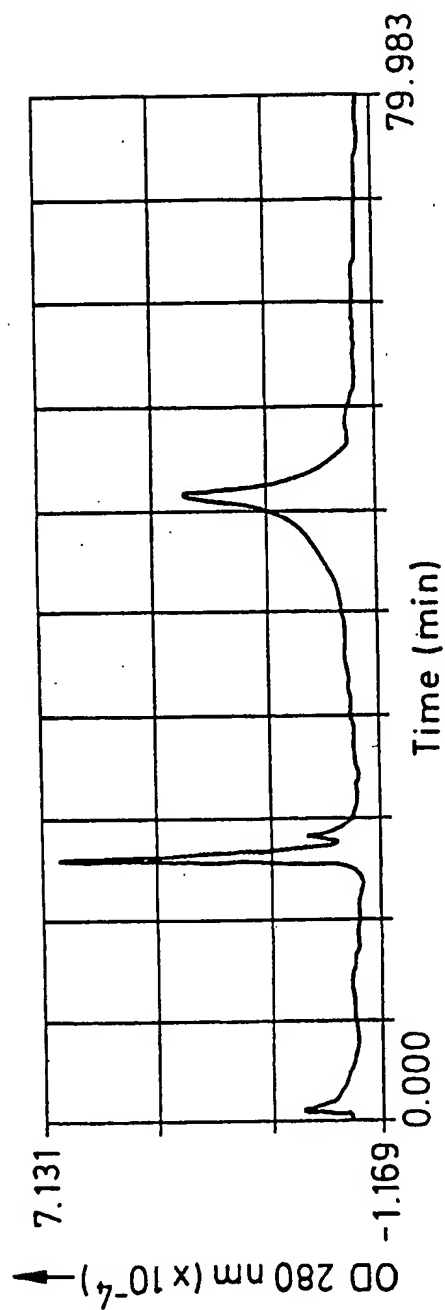


FIG. 1

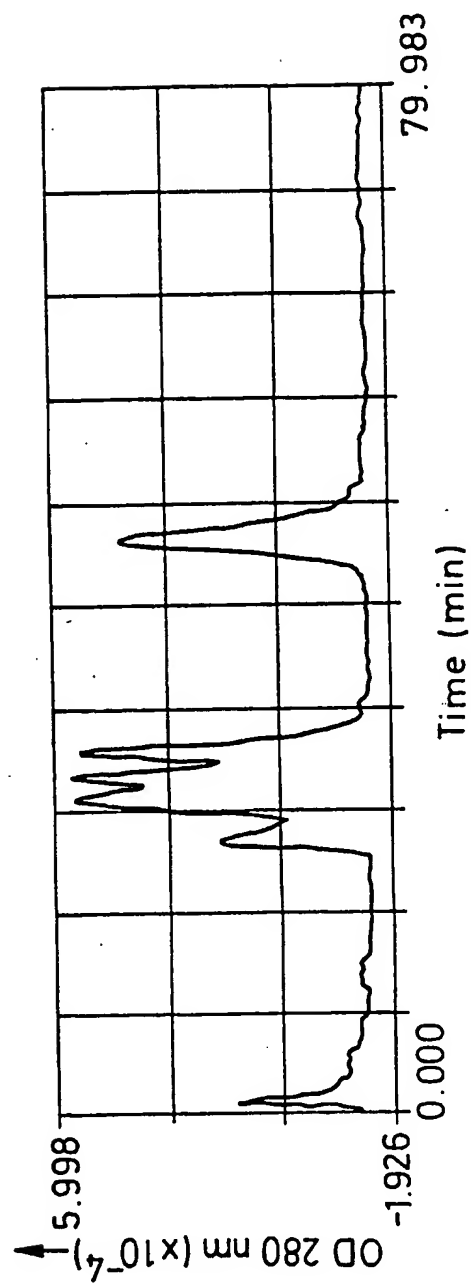


FIG. 2

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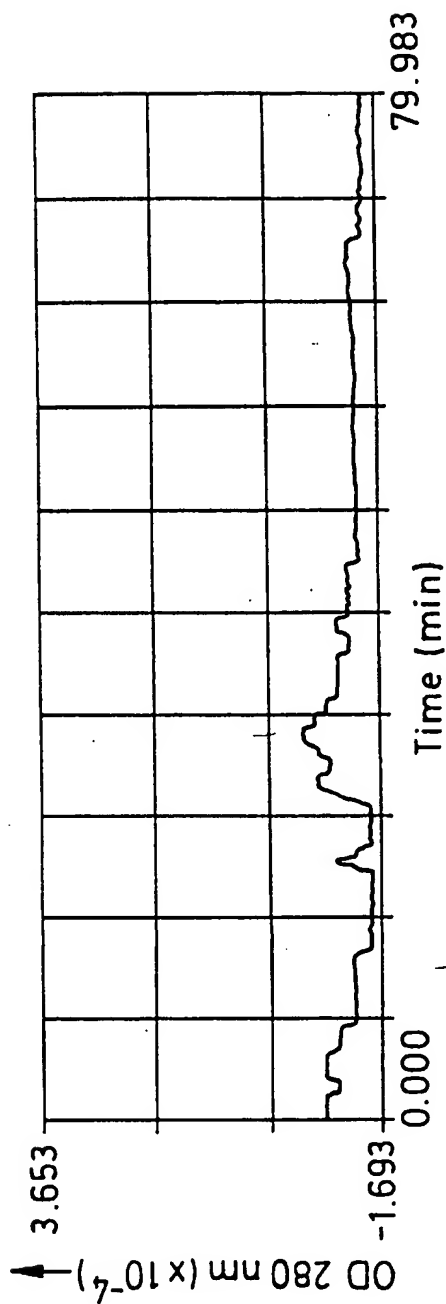


FIG. 3

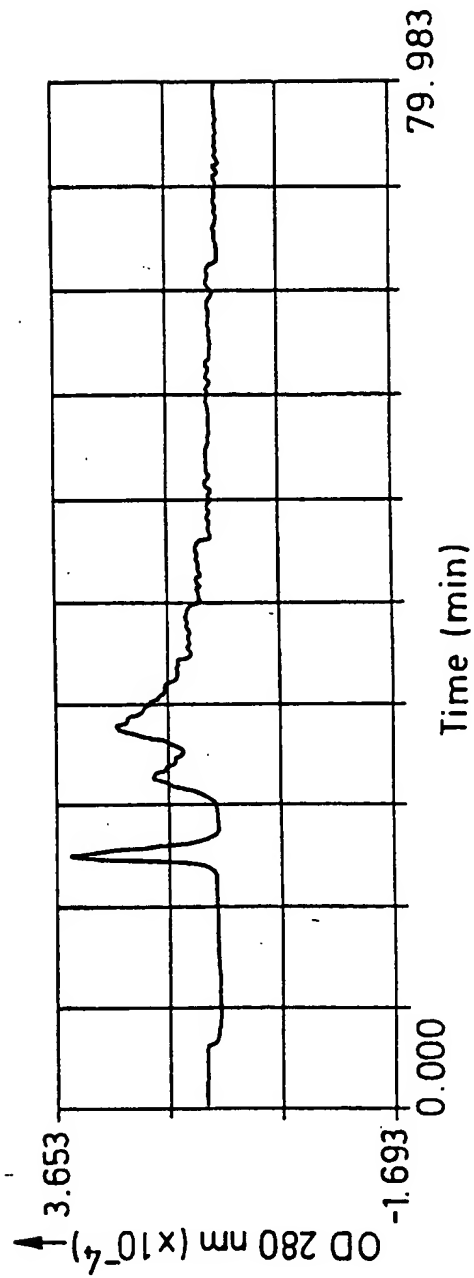


FIG. 4

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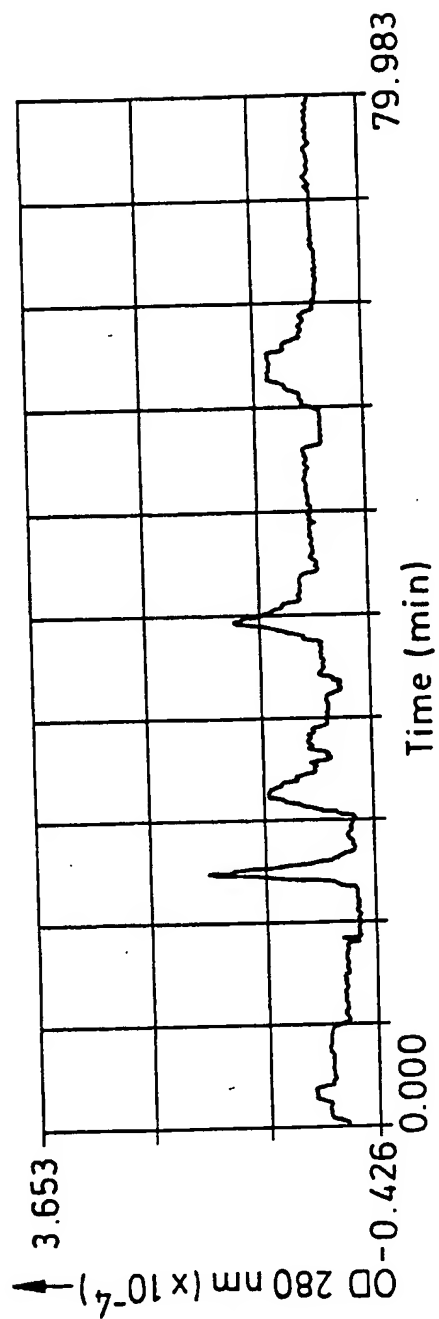


FIG. 5

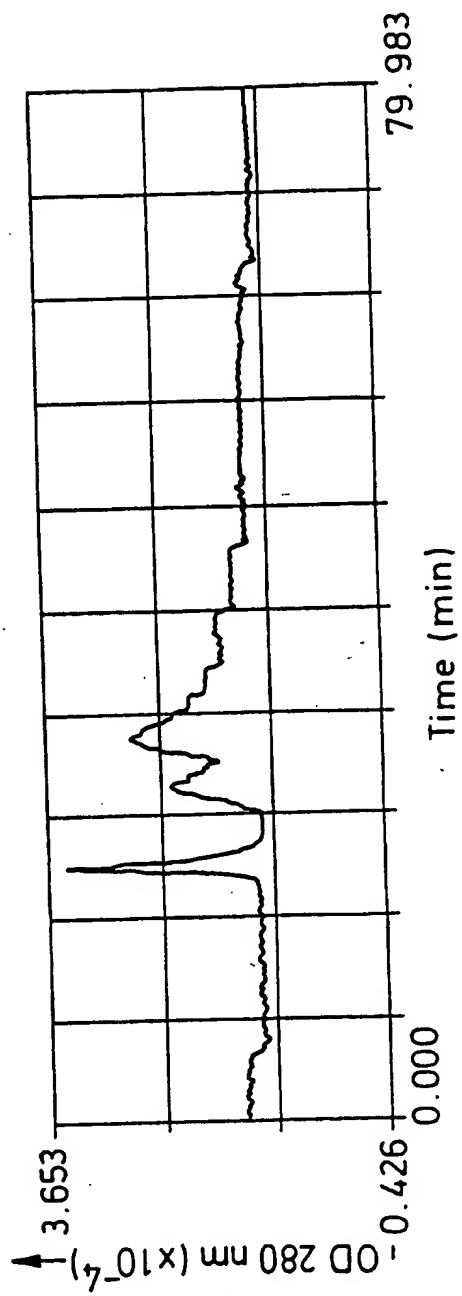
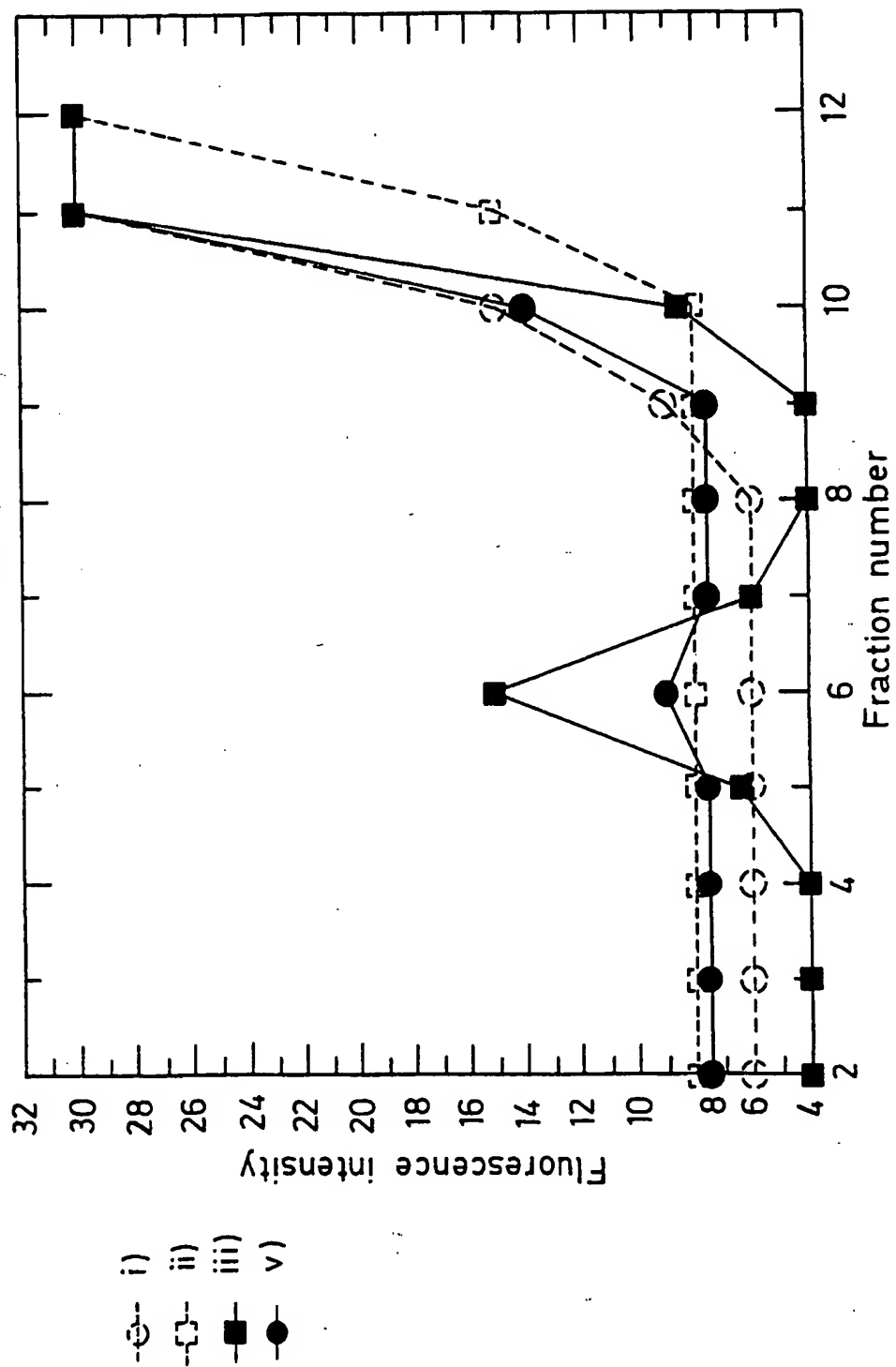


FIG. 6

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FIG. 7



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FIG. 8

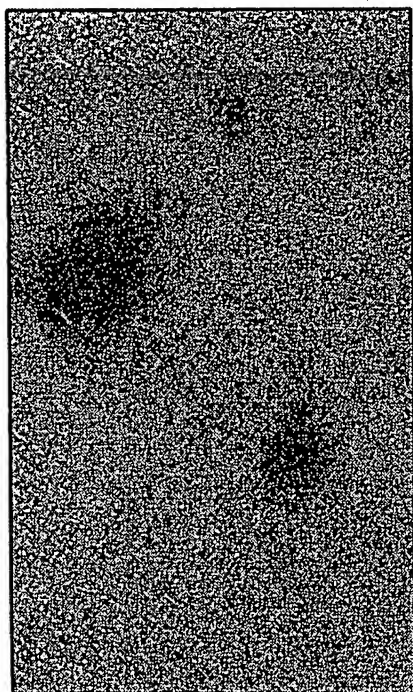
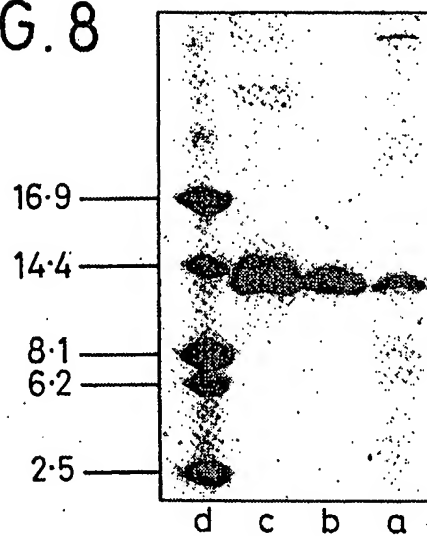


FIG. 9

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FIG. 10

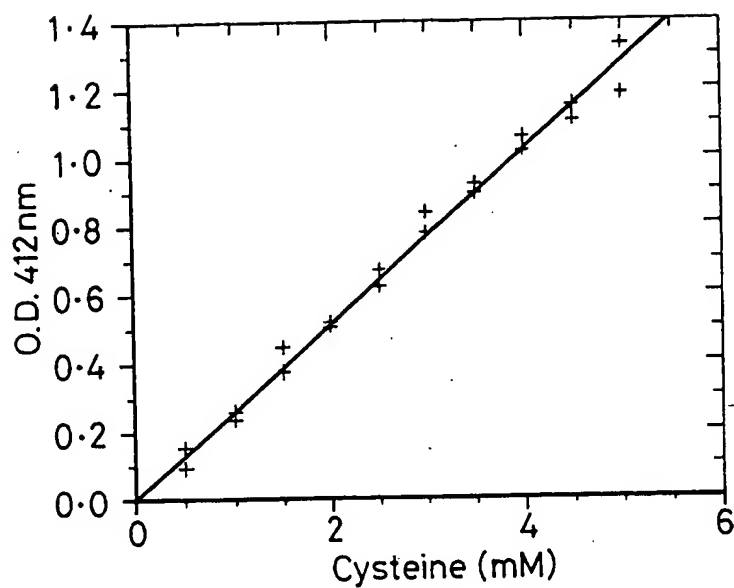
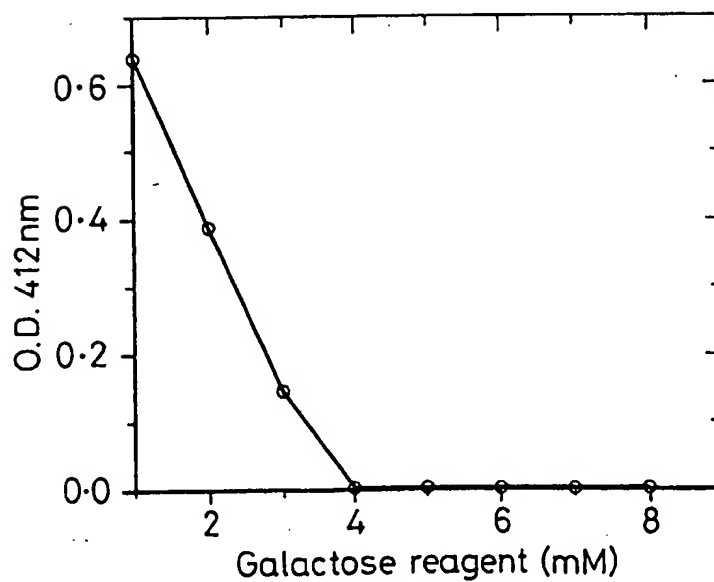


FIG. 11



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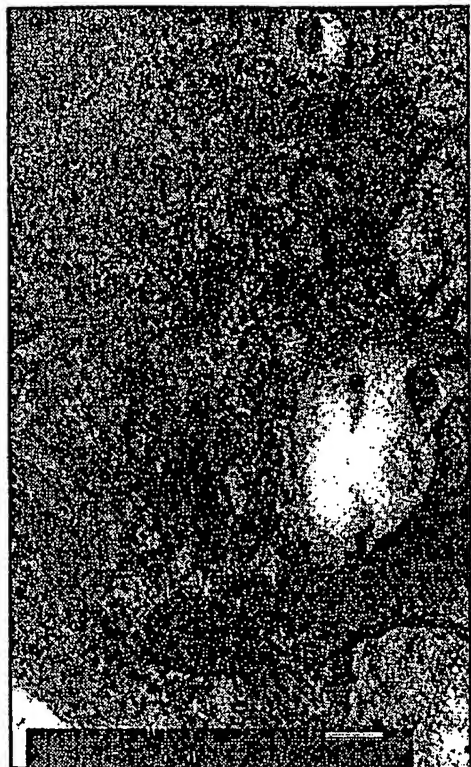
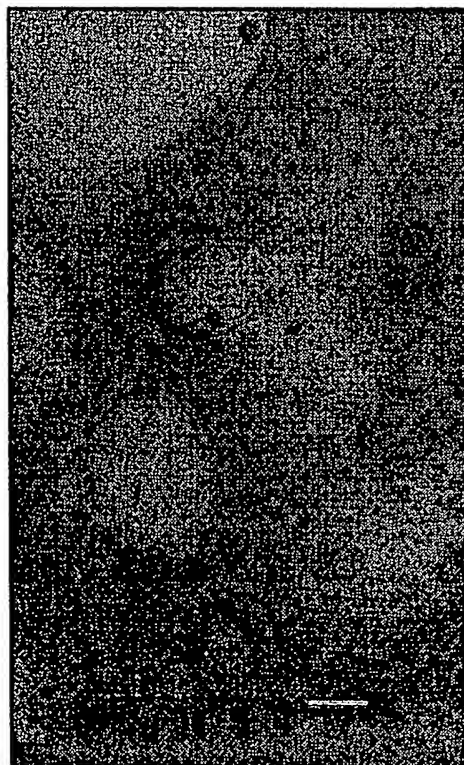


FIG. 12

FIG. 13



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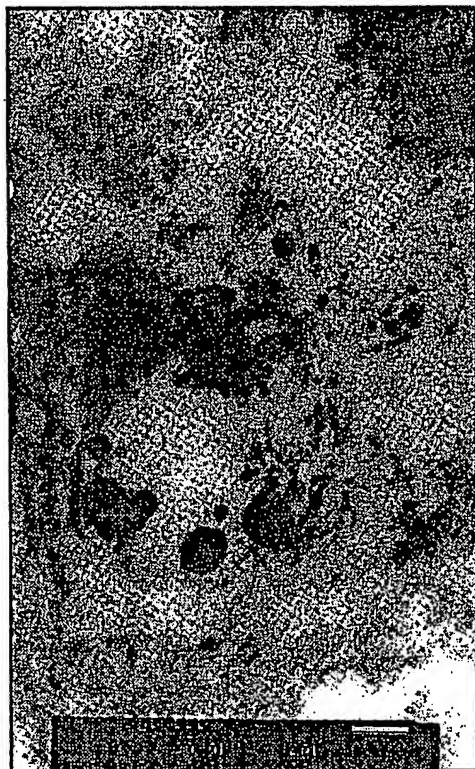


FIG. 14

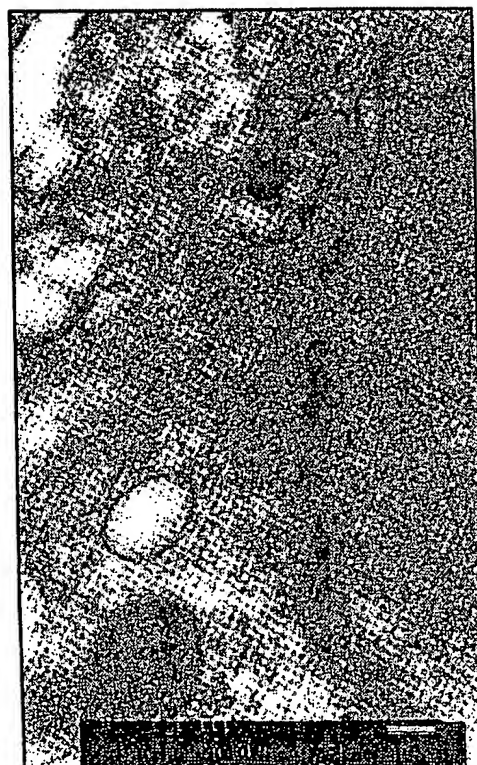


FIG. 15

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 93/01338

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/87; C12N15/11; C12N15/33; A61K9/50		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,8 706 261 (DIATECH LIMITED) 22 October 1987	1,2,11
Y	abstract; claim 4	3-10
Y	WO,A,9 206 180 (UNIVERSITY OF CONNETICUT) 16 April 1992 cited in the application see page 4, line 14 - page 8, line 31	3-11
Y	WO,A,9 203 537 (THERION BIOLOGICS CORPORATION) 5 March 1992 see page 5, paragraph 2 - page 6, paragraph 1	11
-/--		
<p>⁹ Special categories of cited documents:¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
30 AUGUST 1993	20.09.93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	HORNIG H.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category ^a	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	<p>NUCLEIC ACIDS RESEARCH vol. 18, no. 12, 25 June 1990, IRL PRESS, OXFORD, ENGLAND; pages 3521 - 3528 TALBOT ET AL. 'Use of synthetic oligoribonucleotides to probe RNA-protein interactions in the MS2 translational operator complex' cited in the application see page 3526, right column, paragraph 5 - page 3528, left column, paragraph 1 abstract</p> <p style="text-align: center;">---</p>	3,4
P,Y	<p>GB,A,2 257 431 (BRITISH TECHNOLOGY GROUP LIMITED) 13 January 1993 cited in the application see page 11, line 15 - page 13, line 23; claims 1-15</p> <p style="text-align: center;">---</p>	3-11
P,Y	<p>WO,A,9 213 081 (BRITISH TECHNOLOGY GROUP LIMITED) 6 August 1992 cited in the application see page 9, line 15 - page 18, line 16; claims 1-11</p> <p style="text-align: center;">-----</p>	3-11

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9301338
SA 75535

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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30/08/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO-A-9206180	16-04-92	AU-A- 8860391	28-04-92
		CA-A- 2092323	02-04-92
		EP-A- 0553235	04-08-93
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		WO-A- 9300434	07-01-93
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		GB-A- 2253626	16-09-92

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